Noxious Stimulation of the Rat Tooth Pulp May Impair Learning and Memory Through the Induction of Hippocampal Apoptosis

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Aims: To determine whether noxious stimulation of the rat tooth pulp induces learning and memory impairment through the induction of apoptosis in the hippocampus. **Methods:** Thirty-five adult rats were divided randomly into five groups (each n = 7) as follows: control, sham-operated, sham-vehicle, capsaicin-treated, and capsaicin plus ibuprofen-treated group. After preparing dental cavities via cutting 2 mm of the distal extremities of the mandibular incisors, polyethylene crowns were placed on the teeth. Based on the study groups, different injections were administered into the cavities. Nociceptive scores for each block were obtained by measuring the number of seconds that the animal spent rubbing and flicking the lower jaw. After recording the nociceptive behaviors, spatial learning and memory were assessed by using the Morris Water Maze (MWM) test. The hippocampal levels of Bcl-2, Bax, and caspase-3 protein were determined by immunoblotting. Statistical analyses were performed using one- or two-way analysis of variance. **Results:** Noxious pulp stimulation induced by intradental application of capsaicin significantly increased time and traveled distance in the MWM test. Capsaicin stimulation of the pulp also significantly increased the Bax:Bcl-2 ratio and activated caspase-3 in the hippocampus ($P < .01$), which was inhibited by ibuprofen pretreatment ($P < .05$). **Conclusion:** Memory and learning impairment induced by noxious stimulation of the rat tooth pulp may be correlated with activation of apoptotic pathways in the hippocampus.
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Keywords: *apoptosis, capsaicin, hippocampus, learning and memory, tooth pulp*

Odontalgia affects millions of people around the world on a daily basis.[1] Furthermore, orofacial pain involves specific processing pathways and relay sites that make it different from pain originating from tissues supplied by the spinal system.[2] It is speculated that the neural systems involved in cognition and pain processing are closely correlated and that they may modulate one another reciprocally.[3] Pain-induced learning and memory deficits have been reported in several studies.[3–6]

Stimulation of the tooth pulp with formalin or capsaicin may impair cognition in rats, but oral administration of ibuprofen 20 minutes before capsaicin injection can significantly decrease nociceptive scores and cognitive deficits.[7] Ibuprofen might ameliorate inflammation and pain by diminishing the capsaicin-induced proinflammatory mediators that are produced after vanilloid type 1 receptor (VR1) activation.[8] The precise mechanisms of pain-related cognitive impairment have not yet been elucidated, although a number of possible mechanisms have been proposed,[9-12] including hippocampal damage associated with learning and memory impairments.[13] The exact cellular mechanisms underlying the vulnerability of the hippocampus remain to be elucidated, but it is likely that hippocampal vulnerability may result from cellular components involved in hippocampal apoptosis and neurogenesis.[14] Neuronal apoptosis is accomplished by inhibiting the expression of proapoptotic factors (ie, Bax) as well as by promoting the expression of antiapoptotic factors (ie, Bcl-2). In addition, caspase-3 plays a central role in apoptosis and

is considered to be the final executor of the apoptosis pathway. The levels of activated caspase-3, Bax, and Bcl-2 are commonly used as biomarkers for assessment of apoptosis and for clarifying mechanisms of apoptosis induction. Therefore, by focusing on the hippocampus as a key brain area that plays a vital role in learning and memory, the present study explored apoptotic factors in the hippocampus of adult male rats after application of the noxious agent capsaicin to the rat incisor pulp. The aim of the study was to determine whether noxious stimulation of the rat tooth pulp induces learning and memory impairment through the induction of apoptosis in the hippocampus.

Materials and Methods

Animals

Thirty-five adult male Wistar rats weighing 250 to 300 grams, purchased from the Neuroscience Research Center (Kerman University of Medical Sciences, Iran), were used in this study. The rats were housed (12-hour light/dark cycle) one per cage in a room with a temperature of 23 ± 2°C with unlimited access to standard rat chow and water before and during the study. All experimental procedures were approved by the Animal Research Ethics Committee of Kerman University of Medical Sciences, Kerman, Iran (Code: K/90/258).

Dental Procedures

Noxious dental pulp stimulation was based on a modification of the Chidiac et al study[15] described in a previous article.[16] In brief, the distal 2 mm of the rats' mandibular incisors were cut off carefully in such a way that a thin layer of dentin covered the pulp. Then special polyethylene crowns were fixed on the teeth by using a flow composite resin (Tetric Flow, IvoclarVivadent). In contrast to the original model, flow composite was used instead of wire to maximize the retention of crowns and to eliminate the irritation of wire. Moreover, five auxiliary retention holes were added (two in the buccal aspect and one in the lingual and lateral aspects) to the crowns. A small space remained between the tooth structure and the internal surface of the crown.

Drugs

Capsaicin (Sigma-Aldrich) was dissolved in Tween 80-ethanol solution (Merck) (10% ethanol, 10% Tween 80, 80% distilled water, w/w) and administrated intradentally (id) at a dose of 100 µg. It was delivered in a total volume of 10 µL. Ibuprofen (Kimidaru) powder was dissolved in a vehicle (2% Tween 80/distilled water) and given intragastrically (oral gavage) at a dose of 120 mg/kg.[16]

Experimental Design

Thirty-five animals were randomly divided into five experimental groups (each n = 7) as follows:

- Control group; consisted of intact animals
- Sham-operated group; consisted of animals receiving crowns without any injection
- Sham vehicle group; consisted of animals receiving id injection of capsaicin vehicle for 5 consecutive days
- Capsaicin-treated group; consisted of animals receiving capsaicin (100 µg, id) for 5 consecutive days
- Ibuprofen-treated group, consisted of animals receiving ibuprofen (120 mg/kg) 20 minutes before capsaicin injection for 5 consecutive days

Nociceptive Behavior

Test sessions were carried out during the light phase, between 09:00 and 13:00 hours, in a quiet room maintained automatically at 23°C ± 2°C. Before injection of the drugs, each animal was placed in the test box for a 30-minute habituation period to minimize additional stress. The rats did not have access to food or water during the test.

Immediately following the injection, each rat was placed back in the transparent Plexiglas box (25 × 35 × 35) with a transparent floor positioned over a mirror at an angle of 45 degrees to allow for observation of nociceptive behavior. The rats' behavior was observed for 21 minutes within 7 blocks of 3 minutes. Nociceptive scores were determined for each block by measuring the number of seconds that the animal presented each of the following responses, which represents the same scoring criteria as Chidiac et al study[15]: *(0)* calm, normal behavior, such as grooming; *(1)* abnormal head movements, such as mild head shaking or continuous placement of the jaw on the floor or the wall of the cage; *(2)* abnormal continuous shaking of the lower jaw; *(3)* excessive rubbing of the mouth with foreleg movements, such as head grooming, but concentrated consistently and mainly on the lower jaw. A video camera was used to record the behavioral responses.

Morris Water Maze (MWM) Test

The MWM test was used for learning and memory assessment. In brief, it was a black circular pool with a diameter of 136 cm and a height of 60 cm, filled with 20°C ± 1°C water to a depth of 25 cm. The maze was divided geographically into four equal quadrants with release points in each quadrant at north, east, south, and west. A hidden circular platform (10 cm in diameter) made of Plexiglas was located in the center of the southwest (target) quadrant, submerged 1.5 cm beneath

the surface of the water. Fixed, extra-maze visual cues, including highly visible geometric images, were present at various locations around the maze. These consisted of geometric shapes on the walls and shelves, a computer, a window, a door, and posters. These were kept in fixed positions with respect to the swimming pool to allow the rat to locate the escape platform hidden below the water's surface. A video camera was mounted directly above the water maze to record the rat's swim path. A tracking system was used to measure each rat's distance traveled, the percent of distance, and the time in each quadrant. The trials were conducted for 4 consecutive days to observe the rat's escape latency and time spent in each quadrant. The probe trial testing was then performed on the fifth day by removing the platform and allowing each rat to swim freely for 90 seconds. However, if this was not achieved, the rat was guided toward the platform and left there for 20 seconds. All tests were conducted between 09:00 and 13:00 hours.[17]

Tissue Extraction and Preparation

Rats were anesthetized with carbon dioxide and decapitated prior to removal of the brain. Then brains were dissected along the sagittal midline, followed by bilateral removal of the hippocampus.[18] The hippocampus was immediately placed on ice in a glass petri dish. Tissue samples were weighed and immediately frozen in liquid nitrogen and stored at –70°C until assay.[19]

Western Blot (Immunoblot) Analysis

The dissected hippocampal tissues were homogenized using radioimmunoprecipitation assay (RIPA) buffer, containing 10 mM trisaminomethane-HCL (Tris–HCl) (pH 7.4), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% sodium dodecyl sulfate (SDS), 0.1% Na-deoxycholate, 1% nonidet P-40 (NP-40) with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2.5 μ/mL of leupeptin, 10 μg/mL of aprotinin) and 1 mM sodium orthovanadate. The homogenate was centrifuged at 14,000 rpm at 4°C for 15 minutes. The resulting supernatant was retained as the whole cell fraction. Protein concentrations were measured using the Bradford method (Bio-Rad Laboratories). Equal amounts of proteins of the sample were separated according to molecular weight by using SDS–PAGE gel electrophoresis (Cinaclone) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) transferred to polyvinylidene fluoride (PVDF) membrane. After blocking with 5% non-fat dried milk in tris-buffered saline with Tween 20 (TBS-T) buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.1% Tween 20), the membrane was probed for the protein of interest with specific antibodies. All antibodies were diluted in blocking buffer.

A dilute solution of primary antibody to caspase-3 (Cell Signaling Technology, 1:1,000), Bax (21): sc-6236, and Bcl-2 (C-2): sc-7382, 1:1,000 was incubated with the membrane under gentle agitation (overnight at 4°C for caspase-3 and 3 hours at room temperature for Bax and Bcl-2). After washing in TBS-T (three times, 5 minutes), the blots were incubated with a horseradish peroxidase-conjugated secondary antibody (1:15,000, GE Health-care Bio-Sciences). The incubation time was 1 hour for caspase-3 and 1.5 hours for Bax and Bcl-2 proteins at room temperature.

The antibody-antigen complexes were detected using the Electrochemiluminescence (ECL) Advance system and exposed to Lumi-Film chemiluminescent detection film (Roch).[19] Beta-actin was used as a loading control. After the films were digitized, the images of bands were evaluated using Lab Work analyzing software (UVP).[19]

Statistical Analyses

An expert statistical consultant who was unaware of the group assignment carried out the data analyses. Differences between groups regarding nociceptive scores were determined by one-way analysis of variance (ANOVA). Data obtained over the first 4 training days from hidden platform tests were analyzed by two-way ANOVA followed by Tukey's test for multiple comparisons. Data of the fifth day were analyzed by one-way ANOVA. Post-hoc analysis was performed using the Tukey's Honestly Significant Difference (HSD) test, and the significance level was set at $P < .05$ for all analyses performed. Data are presented as mean ± standard error of the mean (SEM). For Bax, Bcl-2, and caspase-3 protein assessments, the results were expressed as mean ± SEM. The differences in the amount of Bax, Bcl-2, and caspase-3 proteins, as well as nociceptive scores between groups, were determined by one-way ANOVA followed by the Newman–Keuls test. The values of Bax, Bcl-2, and caspase-3 and the β-actin bands density obtained from gel analysis and band densitometry were calculated. These values were expressed as a protein/β-actin ratio for each sample. $P < .05$ was considered significant.

Results

Effect of Intradental Application of Capsaicin on Nociceptive Behavior

The mean nociceptive score of the capsaicin-treated rats (1.74 ± 0.1) was found to be significantly higher than that of the ibuprofen-pretreated (0.6 ± 0.1) and sham vehicle (0.43 ± 0.13) rats ($P < .001$) (Fig 1).

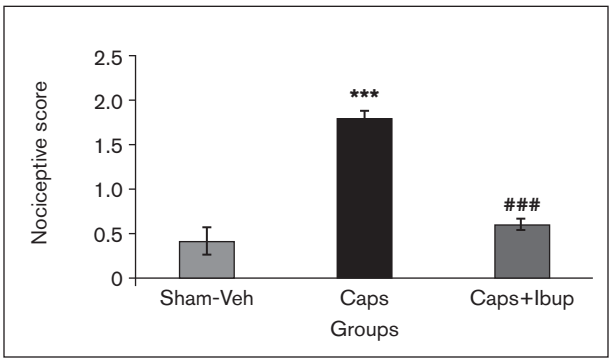

Fig 1 Effect of intradental application of capsaicin on nociceptive scores. Animals were given intradental vehicle (Sham-Veh), 100 µg capsaicin (Caps), or capsaicin plus 120 mg/kg ibuprofen (Caps+Ibup). Two-way analysis of variance used. Each value in the graph represents the mean ± SEM nociceptive scores. ***P < .001 versus sham-vehicle animals. ###P < .001 versus capsaicin-treated animals.

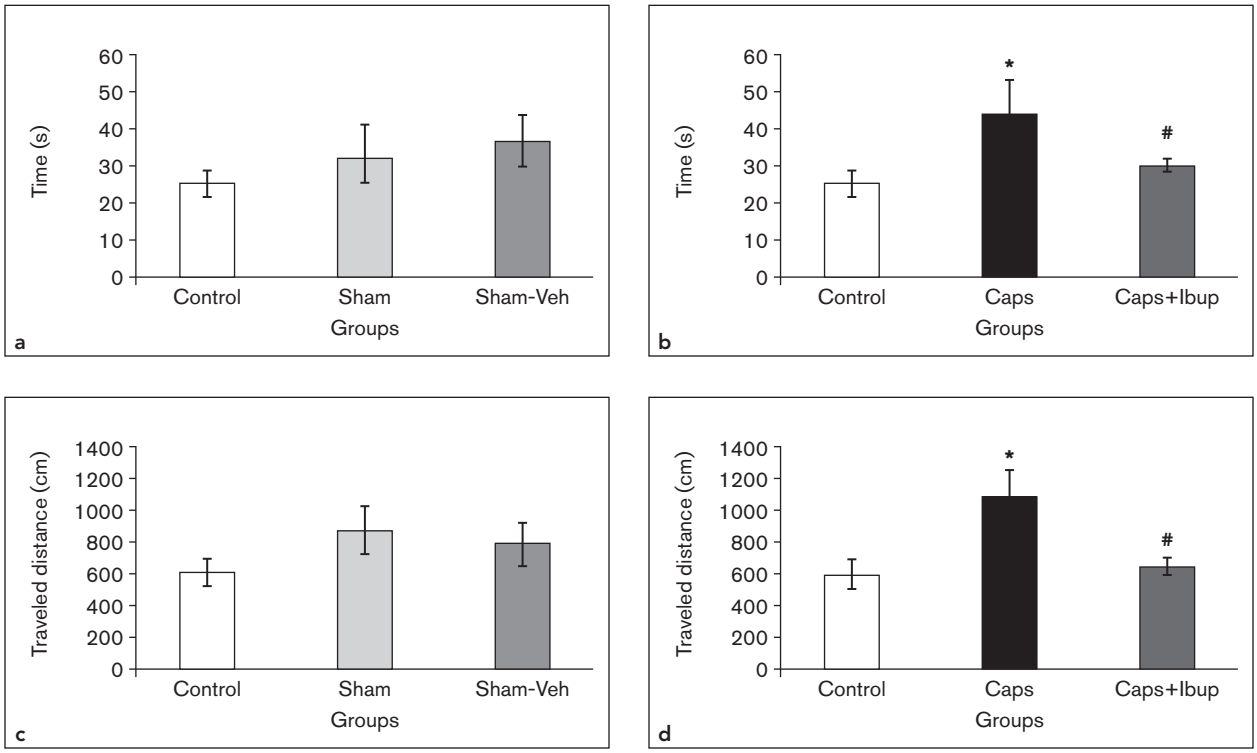

Fig 2 Results of Morris Water Maze test. Comparison of time **(a and b)** and traveled distance **(c and d)** between study groups. Animals were given intradental vehicle (Sham-Veh), 100 µg capsaicin (Caps), or capsaicin plus 120 mg/kg ibuprofen (Caps+Ibup). Two-way analysis of variance used. Values represent mean ± SEM. *P < .05 versus control group. #P < .05 versus capsaicin-treated group.

Effect of Noxious Tooth Pulp Stimulation on Spatial Learning and Memory

Hidden platform trials (days 1–4). There was no significant difference between control, sham-operated, and sham-vehicle groups in time and traveled distance to find the hidden platform (P = .35). Noxious pulp stimulation induced by intradental application of capsaicin (100 µg/rat) significantly increased the time (44.2 ± 8 seconds vs 25.7 ± 2.6 seconds, P < .05) and traveled distance (1,015 ± 85 cm vs 608 ± 107 cm, P < .05) as compared to the control group, while ibuprofen pretreatment prevented these effects (time 30.1 ± 2.5 seconds; distance 608 ± 62 cm) (P < .05) (Fig 2).

Effect of Noxious Tooth Pulp Stimulation on Bax:Bcl-2 Ratio in Hippocampus

There was no significant difference between the control, sham-operated, and sham-vehicle groups in the Bax:Bcl-2 ratio (P > .05). Capsaicin application to

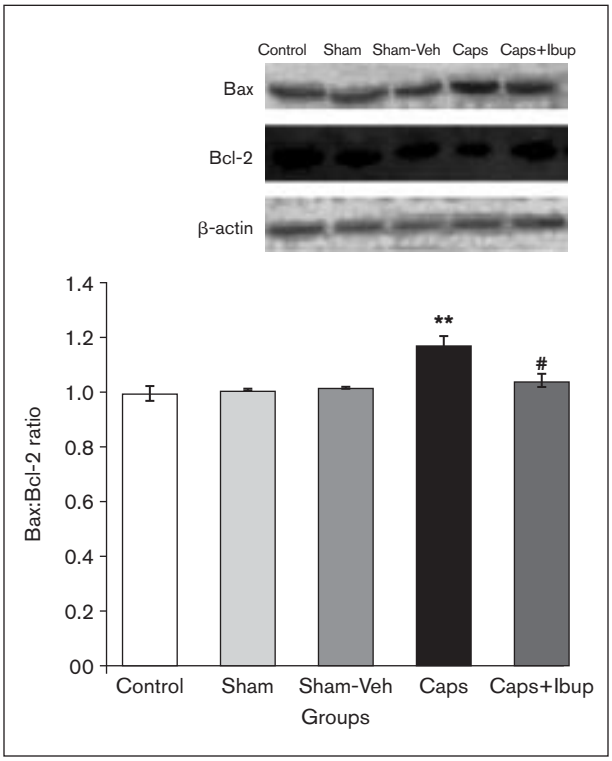

Fig 3 Comparison of Bax:Bcl-2 ratio between study groups. Levels of activated caspase-3 were assayed by Western blotting. β-actin was used as an internal control. Animals were given intradental vehicle (Sham-Veh), 100 μg capsaicin (Caps), or capsaicin plus 120 mg/kg ibuprofen (Caps+Ibup). One-way analysis of variance used. Values represent mean ± SEM. $^{**}P < .01$ versus control, sham, and sham-vehicle groups. $^{\#}P < .05$ versus capsaicin-treated group.

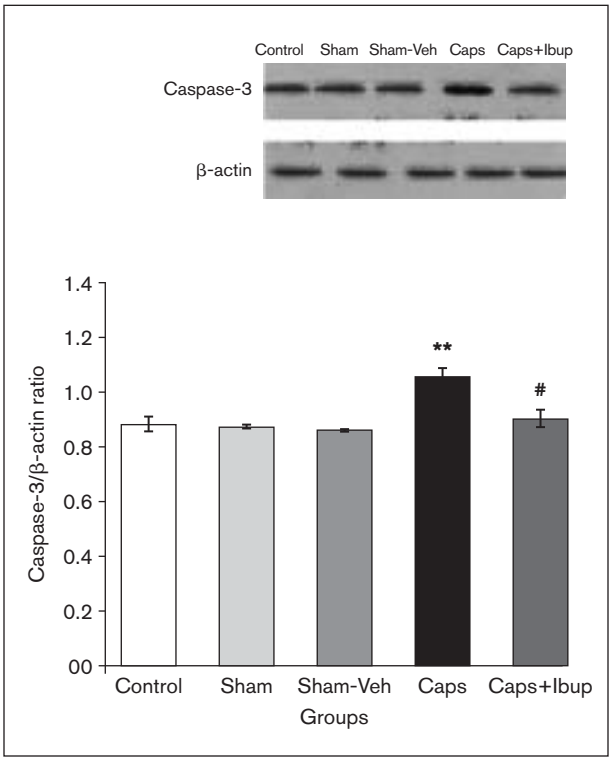

Fig 4 Comparison of caspase-3 protein levels between study groups. Levels of caspase-3 were assayed by Western blotting. β-actin was used as an internal control. Animals were given intradental vehicle (Sham-Veh), 100 μg capsaicin (Caps), or capsaicin plus 120 mg/kg ibuprofen (Caps+Ibup). One-way analysis of variance used. Values represent mean ± SEM. $^{**}P < .01$ versus control, sham, and sham-vehicle groups. $^{\#}P < .05$ versus capsaicin-treated group.

tooth pulp (100 μg/rat) produced an increase in the Bax:Bcl-2 ratio in the hippocampus (1.18 ± 0.061) ($P < .01$). However, in ibuprofen-pretreated rats, Bax:Bcl-2 ratio (1.08 ± 0.05) was close to that in the control (1.01 ± 0.05) groups ($P > .05$) (Fig 3).

Effect of Noxious Tooth Pulp Stimulation on Caspase-3 Protein Level in Hippocampus

There was no significant difference between the control (0.84 ± 0.042), sham-operated, and sham-vehicle groups in caspase-3 protein level in the hippocampus ($P > .05$). Intradental application of capsaicin (100 μg/rat) increased the caspase-3 level (1.08 ± 0.045) in the hippocampus ($P < .01$). The increased caspase-3 protein level was not observed in rats that had capsaicin together with ibuprofen (0.90 ± 0.04) ($P > .05$) (Fig 4).

Discussion

The present study has shown that capsaicin application to the tooth pulp for 5 consecutive days impairs spatial learning and memory of male rats in the MWM test, and that this effect can be prevented by ibuprofen pretreatment. These findings, together with data from other studies,[7,20] support the view that noxious stimulation is associated with impairment of cognitive function. Such an effect, if present in humans, may have a noticeable impact on a patient's quality of life. There is a considerable overlap between the neuroanatomical and neurochemical substrates implicated in both pain and cognition.[2]

Since the hippocampus is closely related to learning and memory, apoptotic cell death of this limbic structure is likely to contribute to cognitive defi-

cits.[21] The production rate of new neurons in the adult mammalian hippocampus is one of the key neurologic processes thought to contribute to cognitive abnormalities.[22] Furthermore, hippocampal neurogenesis can be downregulated by other factors such as persistent pain, stressful conditions, glucocorticoids, and normal aging processes.[22,23]

The present study showed that noxious tooth pulp stimulation induced by repeated intradental injection of capsaicin caused a significant increase in the amount of apoptotic molecular parameters, including the caspase-3 and Bax:Bcl-2 ratio, in the hippocampus. The results also indicated that administration of ibuprofen can prevent the apoptosis of hippocampal neurons. In this regard, Hassanzadeh and Ahmadiani have reported the pain intensity–dependent occurrence of dark neurons in the superficial laminae I–II of the lumbar spinal cord; dark neurons were increased following administration of 5% formalin.[24] The findings of this study are consistent with those of Jalalvand et al, who showed an increased ratio of Bax/Bcl-2 and activated caspase-3 in the hippocampus and dorsal lumbar spinal cord of animals treated with formalin.[25] Furthermore, Joseph and Levine found that caspase-signaling pathways contribute to pain-related behavior in models of painful peripheral neuropathies.[26] Inflammatory and neuropathic pain have been reported to induce cell death in the central nervous system (CNS).[24,27] The hypothalamic-pituitary-adrenal (HPA) axis responds to stressful stimuli, including pain.[28] On the other hand, both acute and chronic stress can impair spatial learning and memory performance.[29] Stress has been known to impair brain function and increase the vulnerability of neurons to injury, especially in the hippocampus,[30] where it induces significant dendritic atrophy and neuronal loss in parallel with impaired spatial memory performance.[31]

Pain may involve increased activation of glutamatergic pathways, leading to excess intracellular Na+ and Ca2+ concentrations and resulting in initial cytotoxic osmotic swelling of neurons and glial cells.[32] Both chronic excitation of neurons involved in nociceptive transmission and the stress caused by pain could be involved in pain-related apoptosis.[25] Moreover, the steroid glucocorticoid hormones released from the adrenal cortex are principal effectors of the stress response.[33] Elevation of glucocorticoids due to sustained stress exerts deleterious effects on the brain.[33] The hippocampus, which has a high density of glucocorticoid receptors, is a principal target of increased levels of glucocorticoids, which induce significant dendritic atrophy and neuronal death in the hippocampus via increased expression of Bcl-2 family genes.[34,35]

Hasegawa et al showed that repeated tooth pulp stimulation has an inhibitory effect on stress responses, represented by a rise in plasma concentrations of catecholamines, corticosterone, and glucose.[36] They suggested that repeated tooth pulp stimulation may exert an antinociceptive effect via activation of an opioidergic descending pain-modulating system. Consistent with this conclusion, it has been demonstrated that the descending systems from the periaqueductal grey (PAG) modulate nociceptive inputs.[37] Furthermore, it has been reported that tooth pulp stimulation strongly activates enkephalinergic neurons and increases the levels of met-enkephalin-like material in the cisternal cerebrospinal fluid of anesthetized cats.[38] In addition, the jaw-opening reflex induced by tooth pulp stimulation can be inhibited by stimulation of the PAG in awake, unrestrained cats.[39,40] Sessle and Hu have also reported that the reflex, as well as trigeminal brainstem sensory neuronal responses elicited by tooth pulp stimulation in rats, can be suppressed by stimulation of the PAG and nucleus raphe magnus, and the effects reversed by an opiate antagonist.[41] The PAG is also involved in cerebral cortical and anterior pretectal nucleus-induced inhibition of the jaw-opening reflex elicited by stimulation of the tooth pulp in rats.[42] In addition, it has been demonstrated that stimulation of tooth pulp increases the release in the cerebrospinal fluid of β-endorphin, which modulates the properties of neurons in the trigeminal brainstem sensory nuclei, interneurons, and motoneurons of the hypoglossal nerve.[43]

The present study had some limitations, mainly its lack of an experimenter blind to the behavioral tests in order to minimize the possibility of experimenter bias. In addition, the main ingredient of both capsaicin and ibuprofen vehicles was Tween 80, but an ibuprofen vehicle group was not utilized for ethical reasons to keep the number of animals used to a minimum. In addition, although the vascular nerve plexus in the rat tooth pulp is similar to that in the human pulp,[44] there are several limitations in relating findings from animal studies to clinical situations in humans; these are well known and include species differences, and thus necessitate that the clinical implications of data obtained from an animal study are cautiously interpreted.

The cognitive impairment observed in the present study may indicate the involvement of pain in the injury of several brain areas including the prefrontal cortex, hippocampus, and amygdala. Future studies should address the vulnerability of these areas to pulp stimulation-induced tissue changes. To develop policy recommendations based on the results of the current study, it is advisable to give verbal postoperative instructions with supplemental written material following a painful dental treatment, since patients may forget what they are told verbally.

Conclusions

Learning and memory deficits have been previously reported in association with different types of pain. However, to the best of the authors' knowledge, this is the first study to show that repeated capsaicin stimulation of the tooth pulp is associated with an increased Bax:Bcl-2 ratio, elevated caspase-3 activity, and neuronal apoptosis in the hippocampus, and that these changes may be functionally correlated with impaired learning and memory.

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